AD_____

Award Number: DAMD17-01-1-0160

TITLE: Metastatic Progression of Breast Cancer by Allelic Loss on Chromosome 18q21

PRINCIPAL INVESTIGATOR: Sam Thiagalingam, Ph.D.

CONTRACTING ORGANIZATION: Boston University
Boston, Massachusetts 02118

REPORT DATE: September 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2004	Annual Summary (1 Sep 2003 - 31 Aug 2004)

4. TITLE AND SUBTITLE
Metastatic Progression of Breast Cancer by Allelic Loss on Chromosome 18q21

5. FUNDING NUMBERS
DAMD17-01-1-0160

6. AUTHOR(S)
Sam Thiagalingam, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Boston University
Boston, Massachusetts 02118

E-Mail: samthia@bu.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The molecular basis of a pro-oncogenic role of TGFβ in promoting advanced metastatic cancer remains unclear. The TGFβ levels are increased locally and systemically in advanced breast tumors particularly at the leading edges and in metastasis. Our studies provide direct evidence for the association between the inactivation of *SMAD4* gene localized to chromosome 18q and upregulation of pro-angiogenic/ metastatic factors such as angiopoietin and VEGF in breast cancer. Additionally, our preliminary data also provides evidence for the synergistic activation of pro-angiogenic/ metastatic effects by TGFβ in the presence of defective *SMAD4* in breast cancer. We therefore hypothesize that normal Smad4 mediated events are required to maintain suppression of metastasis and disabling these events is a major step towards the development of metastatic malignant breast cancer. We are in the process of the identification and characterization of the mediator and effector genes that regulate metastatic progression of breast cancer upon inactivation of the Smad4 mediated events.

14. SUBJECT TERMS
Smad4, metastasis, breast cancer, chromosome 18q, TGFβ

15. NUMBER OF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

ANNUAL REPORT OF THE USAMRMC FUNDED ACTIVITY

Title of the grant: Metastatic progression of breast cancer by allelic loss on chromosome 18q21.

1. Introduction/ Project Overview/ Scientific Progress and future directions:

An association has been established between the high frequency of deletion of chromosome 18q21, where the *SMAD4* gene is localized, with advanced stages of cancers (1). These observations have received added credence from a recent report that suggested up-regulation of metastasis mediator genes such as VEGF and down-regulation of metastatic suppressor genes such as TSP1 in cell lines with *SMAD4* deletions or mutation (2). Our preliminary data very strongly supports these observations and provides the first line of evidence in breast cancer. Additionally, the lack of inactivation of Smad4 at a high frequency in breast tumors prompted us to hypothesize that even though the *SMAD4* gene may not be a direct frequent target for mutational inactivation, the other unidentified target genes in the Smad4 mediated events could potentially be of greater importance in breast cancer due to differences in tissue specificity (1).

Our survey of the various *Smad* genes has provided the first clues in identifying the *Smad8* gene as an important target for loss of expression in nearly 30% of breast cancers which we believe is a significant finding as even the most celebrated tumor marker, *HER/neu* gene amplification, also occurs in about 20%-30% breast cancer cases (3). We found that the inactivation of the *Smad8* gene leading to loss of its expression is mediated by epigenetic DNA methylation (3). It still remains to be determined whether *Smad8* inactivation could also be an alternate target for *Smad2* or *Smad4* inactivation.

Furthermore, our investigations of the consequences of Smad4 inactivation revealed that that lack of Smad4 could favor angiogenesis/ metastasis, which is further enhanced by TGFβ and hypoxia. We are continuing to characterize the cell culture models so that we could undertake comprehensive analysis of the mediator and effector genes, which regulate metastatic progression of breast cancer upon inactivation of the Smad4 signaling pathway.

2. Modified tasks, summary of findings and future directions:

We are making steady progress in increasing the understanding of the implications of the direct or indirect inactivation of *SMAD4* localized to chromosome 18q in metastatic breast cancer.

Task 1. Determination and identification of genetic and epigenetic alterations in known and novel *SMAD*s as potential target genes and the elucidation of their implications to metastatic breast cancer.

With the uses of a novel technique known as TEGD (targeted expressed gene display), we identified that loss of *SMAD8* gene expression could play a major role in the genesis of breast cancer. Our studies provided the first direct clues for epigenetic silencing of *SMAD8* expression due to promoter DNA hypermethylation in breast cancer (3). We have already constructed expression vectors for the *SMAD8* gene. We are in the process of generating defective derivatives of the wildtype *SMAD8* gene as well as planning to undertake siRNA based experiments to dissect the consequences of the *SMAD8* defect in breast cancer. One of the other goals will be to raise monoclonal antibodies against Smad8 to develop potential diagnostic tests. We are currently seeking financial support to take this project forward in a full fledged manner.

Task 2. Identification and elucidation of the roles of alternate target genes involved in the Smad4 signaling pathway.

We have successfully established isogenic cell culture model systems that are proficient and deficient in Smad4 expression. Our data strongly support that the *Smad4* defect favor angiogenesis/ metastasis with significant enhancement in the presence of TGFβ under hypoxic conditions consistent with advanced cancer. Bioinformatics tools will be exploited to construct network of interacting pathways/ events using the various genes identified from the characterization of the model cell lines.

Task 3. Evaluation of candidate target genes.

This task remains unmodified and would begin once we have identified legitimate mediator and effector genes in Tasks 1 & 2. The putative target gene(s) will be evaluated for genetic and epigenetic alterations causing inactivation and/or deregulation in tumors.

Body: Procedures and progress report:

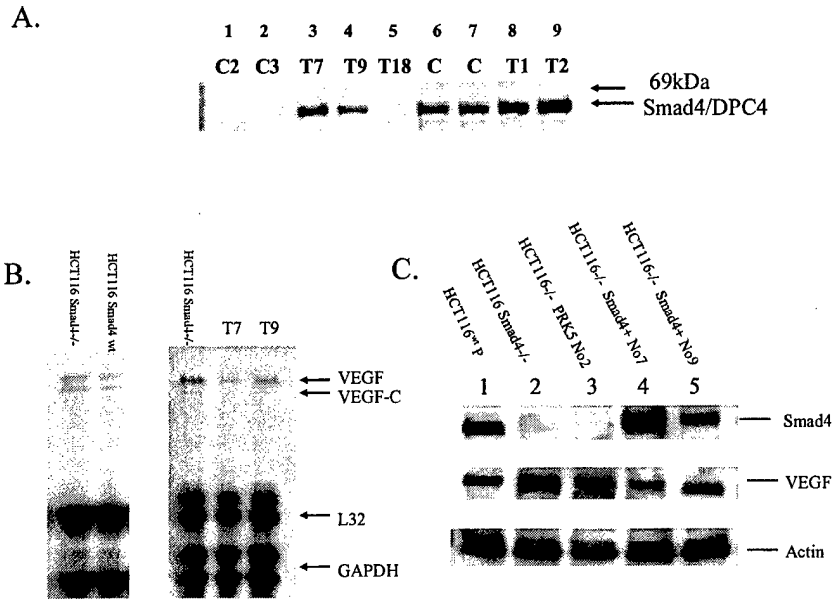

Figure 1. Isolation and initial characterization of *Smad4* stable cell lines.

A. Western blotting was used to screen for stable cell lines that constitutively express *Smad4* and corresponding isogenic controls that have integrated the empty vector. Lanes 1-5 correspond to derivatives of HCT116 Smad4-/- stably transfected with TGFβ-RII receptor and empty

vector (1&2) or pCMV-*Smad4* (3-5). Lanes 6-9 are BR05(*Smad4^{mt}*) stably transfected with an empty vector (6&8) or TGFβ-RII receptor (7) or pCMV-*Smad4* (9). Please note that the clone in lane 5 is a false positive. Although the original HCT116-/- is defective for *Smad4*, BR05(*Smad4^{mt}*) constitutively expresses defective forms of *Smad4*. B. RPA analysis was performed using fifteen μg each of the indicated RNA samples with the RiboQuant multi-probe template set (hAngio-1; BD-PharMingen, San Diego, CA) to detect the indicated mRNAs. L32 and GAPDH were included in each template set as internal controls. C. Western blotting was used to screen for Smad4 and VEGF using β–actin as internal control in original HCT116 Smad4-/-, HCT116 Smad4+/+ and the indicated derivative stable cell lines that either constitutively express or not express Smad4.

The stable cell lines that are proficient and deficient for Smad4 expression showed that over-expression of Smad4 could suppress the expression of pro-angiogenic/ pro-metastatic effectors such as VEGF (Figure 1). Furthermore, we also examined the potential synergistic effects of hypoxia and/or TGFβ itself under the *SMAD4* deficient and proficient conditions (Figure 2). The results from these studies strongly support our hypothesis that the hypoxic conditions and/or the presence of TGFβ had no effect on the levels of VEGF in the presence of abundant levels of Smad4 expression while there was a synergistic enhancement in the levels of VEGF when the cells were defective for Smad4 expression (Figure 2).

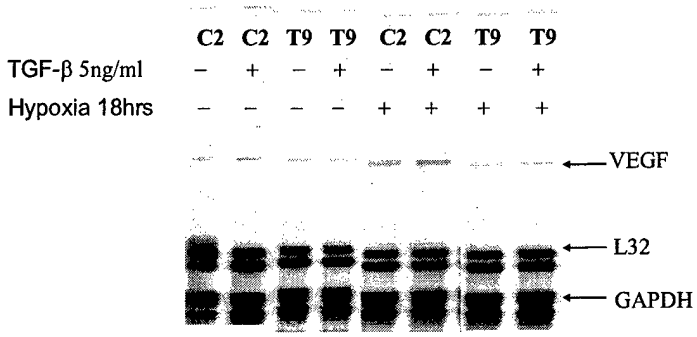

Figure 2. The effect of hypoxia and or TGFβ on VEGF expression in *SMAD4* proficient and deficient cells.

The HCT116 Smad4-/- cell line stably transfected with TGFβ-RII receptor and either empty vector (C2) or pCMV-*Smad4* (T9) were evaluated for VEGF expression in the presence or absence of TGFβ and/ or hypoxia condition (95%N2) by RNase Protection Assay.

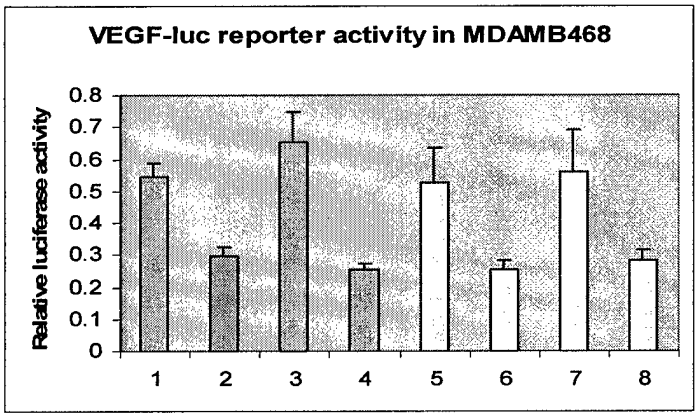

Figure 3. TGF-beta1 and hypoxia enhances the transcriptional activation of VEGF in *SMAD4* defective cells.

The MDAMB 468 Smad4-/- cell line stably transfected with either empty vector (2,3) or pCMV-*Smad4* (7,9), and transiently transfected with the VEGF reporter and Renilla luciferase internal control reporter plasmid. Luciferase activity was measured in cell lysates and plotted as the average with standard deviation for triplicate determinations. VEGF reporter activity was measured in normoxia (green bars) and hypoxia (yellow bars) culture conditions repectively in the indicated Samd4 deficient (2,3) and proficient (7,9) clones.

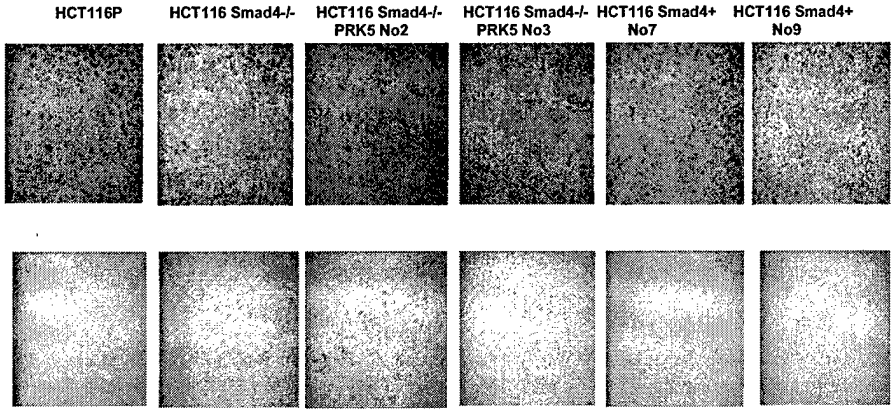

Figure 4. The effects of *SMAD4* status, TGFβ and hypoxia on cell migration.

The indicated cells were plated into the transwell insert with 8.0 um of polycarbonate membrane of a 6-well plate at a density of 2 x10^4 cells/well and cultured 18hrs. After incubation, the cells on the insert were fixed, stained with 0.2% crystal violet, cells on the top of the membrane were photographed and removed with the help of a cotton swab. The cells which have already migrated into the membrane and are not removed by cotton swab were photographed from the bottom of the membrane of the insert. Three fields per insert were selected randomly for photography and all the experiments were independently performed at least twice.

We have further confirmed these observations at the level of gene regulation by performing reporter assays. We chose stable transfectants of the breast cancer cell line MDAMB468 that are either proficient or deficient for *SMAD4* to evaluate the role of Smad4 in the regulation of a pro-angiogenic/ pro-metastatic marker gene VEGF in the presence/ absence of TGFβ as well as normoxic/ hypoxic conditions that mimic the advanced tumors (Figure 3). The hypoxic conditions and/or the presence of TGFβ had little or no effect on the levels of VEGF in the presence of abundant levels of Smad4 expression while there was a synergistic enhancement in the levels of VEGF when the cells were defective for Smad4 expression (Figure 2).

After successfully establishing the regulation of a marker pro-angiogenic and or pro-metastatic factor, VEGF in favor of increased propensity for angiogenesis/ metastasis in Smad4 defective cells, we also found that the defect in Smad4 enabled these cells to exhibit increased motility and migration with synergistic enhancement under hypoxic conditions and in the presence of TGFβ (Figures 4).

In summary, our preliminary data strongly support that Smad4 is a primary suppressor of angiogenesis and or metastasis and the presence of *SMAD4* gene or signaling defect could be the major switch in the conversion of benign tumors to malignancy during multi-step progression of cancer.

We are planning to extend these studies to not only confirm this phenomenon with other candidate genes but also identify a wide spectrum of other critical genes important for the metastatic progression of breast cancer using the microarray (Affymetrix) technology.

Once legitimate metastasis mediator and effecter gene(s) are identified, evaluation of the status of the candidate gene(s) for inactivation/ activation in metastatic breast cancer will commence as described in the original proposal (Task 3).

4. Key research accomplishments:

Our study provide the first direct evidence that 30% of the breast cancers exhibit loss of Smad8 expression and makes it as one of the highly valued markers similar to *Her/neu.* Our studies also provide the first direct evidence that the silencing of gene expression *via* DNA hypermethylation of the *Smad8* gene could be an important event in breast cancer progression and metastasis. Therefore, Smad8 has the potential to become a key target for the development of diagnostic, prognostic and therapeutic strategies to combat breast cancer.

We have also identified/ generated appropriate tumor cell lines as well as experimentally developed derivative test and control cell lines as model systems to identify and isolate the metastatic breast cancer mediator and effector genes involved in the Smad4 signaling pathway.

5. Conclusions:

(1) The loss of *SMAD8* expression in breast cancers is primarily mediated by gene silencing due to epigenetic DNA methylation of regulatory regions.

(2) A combination of *SMAD4* inactivation, high levels of TGFβ and hypoxic conditions could favor angiogenesis/ metastasis.
(3) The identification of target gene(s) that disable Smad4 or Smad8 signaling to promote breast cancer could potentially provide not only novel and valuable diagnostic and prognostic tumor markers but also key arsenals to combat breast cancer.

6. References:

1. Thiagalingam, S., K-h.Cheng, R. L. Foy, H. J. Lee, D. Chinnappan, and J. F. Ponte. 2002. TGFβ and its *Smad* connection to cancer. *Current Genomics* 3: 449-476.
2. Schwarte-Waldhoff I, Volpert OV, Bouck NP, Sipos B, Hahn SA, Klein-Scory S, Luttges J, Kloppel G, Graeven U, Eilert-Micus C, Hintelmann A, and Schmiegel W. 2000. Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. *Pro. Natl. Acad. Sci.* 97: 9624-9629.
3. Cheng, K-h., J. F. Ponte and S. Thiagalingam. 2004. Elucidation of epigenetic inactivation of *SMAD8* in cancer using Targeted Expressed Gene Display. *Cancer Res.* **64**: 1639-1646.

7. Scientific presentation/ publications/ patent relevant to this grant:

Presentations by Dr. Sam Thiagalingam:

The *SMAD* connection to cancer, Genetics and Genomics Friday Seminar, Boston University School of Medicine, January 23, 2004

The *SMAD* connection to cancer, TASME 2004 Conference, Quelph, Canada, July 3, 2004

Publications:

1. Thiagalingam, S., K-h.Cheng, H. J. Lee, N. Mineva, and J. F. Ponte. 2003. Histone deacetylases: Unique players in shaping the epigenetic histone code, *Annal. New York Acad. Sci.* 983: 86-100.

2. Cheng, K-h., J. F. Ponte and S. Thiagalingam. 2004. Elucidation of epigenetic inactivation of *SMAD8* in cancer using Targeted Expressed Gene Display. *Cancer Res.* **64**: 1639-1646.

3. Cheng, K-h., P. Papagiorgis, J. Hunter, M. C. Petriello, J. F. Ponte and S. Thiagalingam. 2004. Smad signaling inactivation promotes cancer metastasis. *Manuscript in preparation.*

Patent:

1. Method of determining gene expression-Targeted Expressed Gene Display – Filed provisional application (BU03-16; March 25, 2003); Boston University.